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A non-thiazolidinedione PPARγ agonist reverses endothelial dysfunction in diabetic (db/db -/-) mice

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PPARγ agonist reverses endothelial dysfunction in db/db mice

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Non standard abbreviations:

COOH: 2-(2-(4-phenoxy-2-propylphenoxy) ethyl)indole-5-acetic acid

PPAR: peroxisome proliferator activated receptor

EDHF: endothelial derived hyperpolarizing factor

eNOS: endothelial nitric oxide synthase

Abstract

We have previously reported that endothelium-dependent relaxation to acetylcholine is impaired in small mesenteric arteries from spontaneously diabetic (db/db) mice. The objective of the present study was to examine the effects of treatment of the db/db and the insulin resistant ob/ob mice with the PPARγ agonist 2-(2-(4-phenoxy-2-propylphenoxy) ethyl)indole-5-acetic acid (COOH). In the db/db model, an 8 week treatment with COOH (30 mg/kg per day) reduced plasma glucose from 48.0 ± 2.5 mM (untreated) to 12.6 ± 1.1 mM. In contrast, plasma glucose was not elevated in untreated ob/ob mice. Relaxation of SMA mediated by acetylcholine was impaired in the untreated db/db diabetic mice (51.7 ± 7.4 % max relaxation, n=6) but not in the ob/ob mice (70.8 ± 8.6% max relaxation, n=3). This impairment was reversed with COOH treatment (86.9 ±0.4 % max relaxation, n=5). Malondialdehyde was elevated in plasma from diabetic db/db mice (13.9± 1.1 vs. 12.0± 0.7 μmoles/ mL), however, when normalized to total cholesterol, no significant differences in the ratio of lipid peroxidation in plasma were identified. Western blot analysis and quantitative PCR for eNOS was performed on the isolated mesenteric vessels and revealed no differences in the relative levels of eNOS expression in diabetic and control animals; in addition, treatment with COOH had no significant effect on eNOS levels in either group. In summary, endothelial dysfunction and hyperglycemia was completely normalized in COOH-treated db/db mice. In contrast, non-hyperglycemic ob/ob mice exhibited normal vasodilatory responses to acetylcholine and consequently COOH treatment had no effect on endothelial function.

Introduction

Vascular disease is the principal cause of morbidity and mortality in patients with type 2 diabetes (Haffner et al., 1998). However, the causative link between diabetes and its vascular complications remains poorly understood.

A significant advance in our understanding of vascular disease in type 2 diabetes has been the development of mouse models. Two such examples of models of insulin resistance are the db/db and ob/ob mouse lines (Leibel, 1997; Coleman, 1982). The db/db mouse is an extensively studied mouse model which spontaneously develops characteristics of type 2 diabetes including obesity, early insulin resistance producing hyperinsulinemia and an eventual beta-cell secretory defect, marked hyperglycemia, and lipid abnormalities (Hofmann et al., 2002a). The db/db phenotype has been linked to a mutation in the leptin receptor of these animals (Leibel, 1997). The ob/ob mouse is similar to the db/db mouse in the development of obesity, hyperinsulinemia and insulin resistance (Haluzik et al., 2004), however hyperglycemia is not prominent. The ob mutation arose spontaneously in the leptin gene of the C57BL/6J mouse (Zhang et al., 1994).

Peroxisome proliferator-activated receptors (PPARs) are ligand activated transcription factors belonging to the nuclear receptor superfamily and have multiple metabolic and cardiovascular effects (Hsueh and Bruemmer, 2004). Several ligands for the PPARγ isoform are clinically available. Thiazolidinedione agonists for PPARγ receptors act as insulin sensitizing agents and are thought to have uniquely beneficial effects on vascular function in diabetics (van Wijk and Rabelink, 2004). Thiazolidinediones have been shown to prevent the development of atherosclerosis in

several experimental models (Li, 2000;Aizawa et al., 2001;Collins et al., 2001). In addition, the thiazolidinedione rosiglitazone has been reported to improve endothelial function in diabetic mice independently of improvements in metabolic dysfunction (Bagi, 2004). COOH is a PPARγ agonist which bears a carboxylic acid pharmacophore in place of the thiazolidinedione moiety found in the PPARγ agonists currently in clinical use. It has previously been shown to act as an insulin sensitizer in a pharmacologically similar manner to the thiazolidinediones (Berger et al., 2001;Laplante et al., 2003).

Endothelial dysfunction can be identified by the reduction in endothelium-dependent vasodilator response to acetylcholine (ACh). Although the endothelium has many additional functions beyond the control of vascular tone, this reduced response to ACh serves as an important indicator of vascular dysfunction and has been closely associated with diabetes in both humans and animals (De Vriese et al., 2000; Pannirselvam et al., 2003). There is increasing evidence of a prognostic link between endothelial dysfunction, as defined by the vasodilator response to ACh, and the later development of vascular complications in both diabetic and non-diabetic populations (Verma et al., 2003; Mancini, 2004), however, the cellular basis for endothelial dysfunction remains unknown.

We have previously demonstrated the development of endothelial dysfunction in the db/db mouse model of type 2 diabetes (Pannirselvam et al., 2002; 2003). In the present study we compared endothelial function in small mesenteric arteries (SMA) from hyperlycaemic db/db mice with SMA from obese, but not overtly hyperglycemic, ob/ob mice and assessed the influence of chronic treatment with the experimental non-thiazolidinedione PPARγ agonist COOH. Our objectives were two fold: firstly to

determine whether correction of endothelial dysfunction by PPARγ agonists extended to non-thiazolinedione members of this class of agents; and secondly, to examine potential molecular targets within the vasculature that may be involved in mediating endothelial dysfunction and the response to the PPARγ class of drugs.

Methods

Drug Treatment

Male C57BL/KsJ-lepr^{db}/lepr^{db} (db/db) mice (18 animals), male C57BL/6J-lep^{ob}/lep^{ob} (ob/ob) mice (9 animals) and respective age-matched controls (db/+ and ob/+) were obtained from Jackson Laboratories (Bar Harbour, ME). At 8 weeks of age each type of animal was divided into three equal groups. Group 1 received 30 mg/kg/day of COOH (*2-(2-(4-phenoxy-2-propylphenoxy) ethyl)indole-5-acetic acid*) in powder chow (Carley et al., 2004) for 8 weeks. Group 2 received regular powdered chow (ProLab RMH 2500/5P14; PMI International, Brentwood, MO) for 8 weeks. Group 3 received 30mg/kg/day of COOH for 8 weeks and then were crossed over to untreated feed for a further 5 weeks. At the end of the treatment period the animals were killed by cervical dislocation in accordance with a protocol approved by Animal Care Committee at the University of Calgary.

Experimental Protocols

Second and third-order mesenteric arteries were cut into 2 mm rings and mounted on a Mulvany-Halpern myograph (Mulvany and Halpern, 1977). After a 45-minute equilibration period in Carbogen (95% O_2, 5% CO_2) aerated Krebs solution, the vascular reactivity to the alpha adrenoceptor agonist phenylephrine (PE) was studied. Following a 30 minute stabilization period, endothelium-dependent vascular relaxation to ACh was recorded in preparations contracted with a sub-maximal concentration of PE (EC_{75-80}). A repeated 30 minute stabilization period was followed with measurement of relaxation to sodium nitroprusside (SNP) in similarly contracted SMA tissues from db/db, ob/ob and respective controls. PE-induced contractions were normalized to percentage of 120 mM KCl induced contraction.

Plasma glucose, triglyceride and cholesterol were assayed using commercial kits (Sigma & Co., USA). Samples of mesenteric arteries and aorta were flash frozen and later used for RNA extraction.

Quantitative PCR

Total RNA was extracted from mesenteric arteries and aortic tissue using an RNeasy Mini Kit with on-column DNase treatment (Qiagen) and first strand cDNA was subsequently synthesized using a Superscript RT Kit (Qiagen). Real-time PCR primers were designed (beta-actin F: 5' ACGGCCAGGTCATCACTATTG 3'; beta-actin R: 5' CCAAGAAGGAAGGCTGGAAAAGA 3'; eNOS F: 5'

CAACGCTACCACGAGGACA 3'; eNOS R: 5' CTCCTGCAAAGAAAAGCTCTGG 3') and analyzed in positive and negative control PCR reactions with SYBR-green (Qiagen) at 8 annealing temperatures, ranging from 52ºC to 62ºC. Melt curve analysis was performed to visualize primer specificity by revealing the presence or absence of primer dimers. For further verification, 1.0 µL of the PCR reaction was analyzed on an Agilent Technologies 2100 Bioanalyzer using a DNA 500 LabChip kit. Sequencing was performed on products from successful reactions. PCR efficiency was determined by performing real-time PCR on serial dilutions of mouse heart cDNA (94.6% for beta-actin, 96.7% for eNOS). Real-time PCR reactions were carried out using 2.0 µL of first strand cDNA in a total reaction volume of 25 µL containing 1X QuantiTect SYBR Green Supermix (Qiagen) and 0.25 µM forward and reverse primers. PCR reactions were hot started (95ºC for 15 min) and then exposed to 40 cycles of 94ºC for 0.25 min, 55.7ºC for 0.5 min and 72ºC for 0.5 min, where fluorescence data collection occurred during each extension phase. Melt curve analysis was again performed following cycling as a method of validation. The fold relative to beta-actin for eNOS was calculated using the $2^{-\Delta C_T}$ method (Livak and Schmittgen, 2001).

Western Blotting

Homogenates of thoracic aorta were prepared from flash frozen tissue in lysis buffer containing 100 mM Hepes (pH 7.4), 150 mM NaCl, 1 mM $CaCl_2$, 1 mM $MgCl_2$, 0.1 % Tween 20, 1% triton-X 100, and Protease Inhibitor cocktail (Sigma, St. Louis MO). Approximately 50mg of tissue was combined with 0.1 ml of lysis buffer and ground with

a micropestle as well as subjected to three rapid freeze/ thaw cycles to disrupt tissue. Once completely lysed, solution was centrifuged at 13 000 RPM for 10 minutes to remove insoluble material. Protein concentration was determined for each sample using a Bio Rad protein analysis kit. Equal amounts of protein were resolved under reducing conditions on an 8% SDS polyacrylamide gel. Immunoblotting was performed with a polyclonal antibody to eNOS (BD Transduction Laboratories) at a dilution of 1:500 in nonfat milk/ Tris buffer. The membrane was subsequently probed with a secondary anti-rabbit antibody conjugated to horseradish peroxidase at a dilution of 1:1000 and developed with chemiluminescence (Pierce, Rockford, IL). The membrane was then exposed to X-ray film (Kodak) which was subsequently developed.

Statistical Analysis

In all experiments, n equals the number of animals used in the protocol. Values are mean ± standard error of mean. Statistical significance of difference between means of different groups were performed using either Student t test or two-way ANOVA with Bonferroni post-hoc test. A value of $P < 0.05$ was considered statistically significant.

Results

The monogenic murine models of type 2 diabetes used in this study have mutations in the leptin receptor (db/db) and leptin molecule (ob/ob), on genetic

backgrounds predisposed to development of insulin resistance (*C57BL/KsJ* and *C57BL/6J* respectively). Loss of the leptin response axis in both ob/ob and db/db mice leads to chronic hyperphagia and resultant obesity. We observed increased body weights in db/db mice (mean 51.5g vs. 30g db/+; $p<0.05$), and ob/ob mice (mean 60.7g vs. 30.2g ob/+; $p<0.05$) at 16 weeks of age (Table 1). This obesity was accompanied by marked hyperglycemia in untreated db/db mice (48.0 ± 2.5 mM) compared to untreated control db/+ mice (12.3 ± 0.8 mM) (Figure 1). In contrast, the ob/ob mouse displayed no significant change in plasma glucose at 16 weeks of age (16.6 ± 2.4 mM vs. 13.8 ± 2.0 mM in control ob/+ mice). Both the db/db and ob/ob mice showed an increase in total cholesterol relative to their aged matched controls: 2.77 vs. 1.81 mM in the db/db vs. db/+ and 4.30 vs. 2.06 mM in the ob/ob vs. ob/+ (Table 1). Similarly, triglycerides were elevated in db/db mice (2.11 vs. 1.18 mM) but not in ob/ob mice (1.60 vs. 1.53 mM) relative to control animals.

Treatment with COOH, a PPARγ receptor agonist, completely corrected the hyperglycemia seen in the db/db mice (from 48 mM to 13 mM; Figure 1) and significantly decreased cholesterol in the control db/+ group and triglyceride levels in all groups of animals except the control db/+ group (Table 1). When COOH was discontinued, and animals consumed a normal diet for a further five weeks (crossover, Figure 1), the db/db mice became hyperglycemic again, indicating that continued treatment with COOH was required to maintain normoglycemia. No significant difference in glucose levels were noted in the ob/ob mice with treatment, however, these animals are relatively euglycemic without treatment. Malondialdehyde, a marker of lipid peroxidation, was elevated in plasma from the db/db mice (13.9± 1.1 vs. 9.0± 1.2

umoles/mL in db/+), and ob/ob mice (22.9 ± 5.2 vs 8.7± 1.2 umol/mL in ob/+) however, when these results were normalized to total cholesterol in serum, no significant difference in the amount of malondialdehyde per unit cholesterol were seen between treated and untreated animals (Table 1).

Endothelial and vascular function was analysed using the Mulvany-Halparen wire myograph technique on second and third order mesenteric arteries (SMA) from each of the animals. Data is presented in summary graph form. Phenylephrine applied in cumulative concentrations until maximal contractions were achieved (Figure 2A) showed no significant difference between either treated or untreated db/db or ob/ob mice. In contrast, there was an impairment of the relaxation of the SMA mediated by acetylcholine (Figure 2B) in the untreated db/db diabetic mice (51.7 ± 7.4 %max relaxation, pEC50 6.75, n=6) versus their age matched control animals (db/+; 86.1 ± 5.6% max relaxation, pEC50 6.33 n=6; $P< 0.05$). This impairment (endothelial dysfunction) was reversed in the COOH-treated db/db animals (86.9 ±0.4 % max relaxation, pEC50 6.87, n=5). The ob/ob mouse model did not demonstrate a statistically significant impairment of ACh mediated relaxation (70.9% max relaxation, pEC50 6.51; Figure 2B) when compared with its control littermates (69.5% max relaxation, pEC50 6.14; Figure 2B), the experimental number in the ob group was smaller (n=3). No significant differences between any of the groups were observed in relaxation of SMA by the endothelium independent agent sodium nitroprusside (Figure 2C).

Quantitative PCR was performed on flash frozen samples of both aorta and mesenteric arteries for each of the animals to determine if COOH had an effect on eNOS gene transcription. No significant differences were observed in eNOS gene expression

between treated and untreated animals in either the db/db or ob/ob mouse models when results were normalized to beta-actin expression (Figure 3). Expression of eNOS in the ob/+ and ob/ob mouse aorta and mesenteric arteries demonstrated statistically significant elevation in mean level of message compared with their db/+ and db/db counterparts. The relatively lower level of eNOS expression per unit beta-actin observed in the aortic tissues is likely accounted for by the much increased quantity of smooth muscle in aortic samples relative to the eNOS synthesizing endothelial cell layer, thus decreasing the relative amount of eNOS (endothelial cell derived) per unit beta-actin (endothelial and smooth muscle cell derived).

Thoracic aorta tissue samples from db/db and db/+ animals in each of the treatment groups were recovered and analyzed for eNOS protein content using a commercially available polyclonal antibody. Figure 4 presents a representative western blot. Lanes were loaded with equivalent quantities of total protein. Six sets of samples were blotted, and no differences in eNOS protein levels were noted by band densitometry, either between untreated db/db (183 +/-30) and db/+ animals (173 +/- 39) or COOH treated animals (db/db, 171 +/- 29; db/+, 170 +/- 41). Thus eNOS protein and mRNA levels do not appear to be altered by COOH treatment in the db mouse.

Discussion

We have examined the effect of a novel non-thiazolidinedione PPARγ agonist, COOH, on endothelial function in murine models of type 2 diabetes and obesity. The new finding is that treatment with COOH prevents the development of endothelial dysfunction

in the db/db model, as defined by decreased vasodilator responsiveness to ACh. This data supports the hypothesis that the improvement in endothelial function seen with thiazolidinedione PPARγ agonists (Phillips et al., 2003; Li et al., 2000;Aizawa et al., 2001;Collins et al., 2001) is mediated by PPARγ activation that is not specific to the thiazolidinedione class of molecules.

We studied two monogenic mouse models of obesity and metabolic dysfunction in this study. The diabetic db/db mouse is a well accepted model of type 2 diabetes (Coleman, 1982) whereas the ob/ob mouse is a model of obesity and insulin resistance without overt hyperglycemia (Coleman, 1982;Haluzik et al., 2004). Both of the mouse models studied develop metabolic dysfunction as a result of monogenic mutations in the leptin endocrine axis; the db/db mouse is deficient in the ObRb leptin receptor 2B, and the ob/ob mouse is deficient in the leptin molecule itself. However, it is the polygenic background of these animals that largely contributes to the development of insulin resistance (Haluzik et al., 2004). The db/db mouse, on the C57BL/KsJ background, develops marked insulin resistance and then declining insulin levels due to a pancreatic beta-cell secretory defect, producing severe hyperglycemia by 16 weeks of age. The ob/ob mouse, on the C57BL/6J background, exhibits marked obesity and lipid abnormalities without significant hyperglycemia at the same age of 16 weeks. Serum insulin levels have been previously been reported for the C57/BL/KsJ db/db mouse (Hofmann et al., 2002b) showing increases in circulating insulin levels relative to control animals until approximately 14 weeks of age, and then a progressive decline. These data are in accordance with other published data regarding the db/db and ob/ob mice on the *C57BL/KsJ* and *C57BL/6J* backgrounds respectively (Haluzik et al., 2004;Coleman,

1982). However, only the db/db mouse model demonstrated marked endothelial dysfunction in the SMA. This suggests that endothelial dysfunction in the diabetic db/db animals is more closely linked to hyperglycemia than to alterations in the lipid profile.

The marked hyperglycemia in untreated db/db mice was normalized by chronic (8 wk) treatment with COOH. This glucose-lowering effect of COOH is consistent with the observation that insulin-stimulated glucose uptake was enhanced in cardiomyocytes from COOH-treated db/db mice (Carley et al., 2004). Remarkably, the endothelial dysfunction which characterized SMA from untreated db/db mice was completely normalized by COOH treatment. Thus, our study suggests that the prevention of endothelial dysfunction with COOH treatment may be secondary to the improvement of the metabolic profile of the db/db animals, most likely due to correction of chronic hyperglycemia. (Bagi et al., 2004), however, have reported that a one week treatment of db/db mice with rosiglitazone reducd oxidative stress and reversed endothelial dysfunction in the coronary arteries of db/db mice without any significant action on the metabolic abnormalities, thus indicating that the improvement of endothelial function, at least with rosiglitazone, is independent of changes in insulin sensitivity. Further studies are required to investigate the direct effects of PPARγ activation on endothelial function.

The key source of NO for NO-mediated relaxation in the endothelium is thought to be the enzyme endothelial nitric oxide synthase (eNOS). While post translational modification and co-factors play a major role in regulation of this enzyme, its production in the endothelium is critical to normal endothelial function (Andrews et al., 2005; Verma et al., 2003). Using quantitative PCR techniques and Western blotting we examined eNOS message and protein levels in endothelium of arterial tissue. Although we found

significant differences in eNOS expression between the db/db and ob/ob mouse models, we did not observe an effect of COOH on eNOS gene expression or protein content. This suggests that COOH does not directly effect eNOS gene transcription. However, the increased expression of eNOS in the ob/ob mice, relative to that determined in db/db and control mice, may have a protective effect inhibiting the development of endothelial dysfunction in these animals that may result from the dyslipidemic state of the ob/ob mouse.

PPARγ activation stimulates fatty acid storage in adipose tissue. Its activation results in both an increase in adipocyte number and fatty acid influx into adipocytes, and a remodeling of adipose tissue (Ferre, 2004). In addition, COOH has been shown to alter the distribution of adiposity in rodents (Laplante et al., 2003). In this study we note that additional weight was gained by those animals treated with COOH (Table 1). Thus, the glucose lowering effect of PPARγ agonists due to insulin sensitization may be due largely to adipose tissue actions, a proposition supported by studies performed with tissue-specific PPARγ null mice (Evans et al., 2004). While it is tempting to ascribe all the actions of COOH to its role in regulating adipose tissue metabolism, PPARγ agonists have pleiotropic effects including stimulation insulin dependent glucose transporter GLUT4, upregulation of the angiopoietin related gene PGAR, and adiponectin as well as direct actions in vascular smooth muscle (Bruemmer et al., 2003a; 2003 b) The antiatherosclerotic actions of the PPARγ agonists (see Berger et al., 2005) are also suggestive that these drugs are also targeting specific endothelial cell genes.

Reactive oxygen species (ROS) are a family of molecules, including molecular oxygen and its derivatives, which are produced in all aerobic cells. NO is an important

tonic inhibitory factor for controlling mitochondrial respiration and thus a decrease in eNOS activity (or NO bioavailability) will result in an increase in superoxide production by mitochondria. Brownlee and colleagues (Nishikawa et al., 2000) have argued, based on studies with cultured endothelial cells, that mitochondria are the source of ROS and observed that uncoupling oxidative phosphorylation in bovine aortic endothelial cells that had been treated with high glucose prevents the sequelae of hyperglycemia. Unfortunately intervention studies in humans with antioxidants (notably vitamin C and/or E) have provided confusing and conflicting results. Data from studies with the db/db mouse indicate that acute incubation with indomethacin, a non-selective inhibitor of COX, and SQ29548, a selective thromboxane receptor antagonist, significantly attenuated the enhanced contraction to α-adrenoceptor agonists in the SMA and thus enhanced thromboxane generation may also contribute to vascular dysfunction in diabetes (Pannirselvam et al., 2005). We thus examined lipid peroxidation products in the serum of our animals using an assay for malondialdehyde. Malondialdehyde is derived from peroxidation of fatty acid chains and has been proposed to provide an approximation of oxidative stress (Nielsen et al., 1997). While plasma malondialdehyde levels were higher in the db/db and ob/ob animals compared with controls, we found no significant differences in the amount of malondialdehyde per unit cholesterol. We chose to undertake the normalization to serum cholesterol in order to account for the significant differences in serum lipid content between the different animals. Our assumption is that a greater content of fatty acids in the serum results in an increase in malondialdehyde levels independent of changes in oxidative stress. While this normalization suggests that there is not a marked elevation in overall oxidative stress in these murine models,

malondialdehyde levels provide only a gross measure of overall oxidative stress in the animals and does not rule out the possibility of an increase in oxidative stress within the endothelium itself (Halliwell and Whiteman, 2004). We have reported, based on the use of dihydroethidium as a fluorescent dye indicator of oxidative stress, that intracellular superoxide levels are elevated in *in situ* frozen unfixed sections of SMA from 16 week male (untreated) db/db mice (Pannirselvam et al., 2005) thus suggesting that it is the elevated intracellular oxidative stress that may determine endothelial dysfunction.

The development of peripheral edema is a major side effect limiting the usefulness of currently available PPARγ agonists, affecting approximately 5% of patients on a thiazolidinedione and 15% of patients who combine thiazolidinediones with insulin therapy (Mudaliar et al., 2003; King and Levi, 2004). Thus, there has been great interest in the development of new PPARγ ligands that minimize these side effects. We have demonstrated that the non-thiazolidinedione PPARγ agonist COOH reverses the endothelial dysfunction that develops in the db/db mouse model of type 2 diabetes, without any change in eNOS expression. In addition, we demonstrated that endothelial dysfunction does not occur in the non-hyperglycemic ob/ob mouse model and that COOH-treated ob/ob mice show no change in endothelial function. This selective modulatory effect of COOH upon endothelial function may be a reflection of the reversal of hyperglycemia in db/db mice.

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Footnotes

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Legends for Figures

Figure 1 Plasma Glucose measurements from mice prior to sacrifice. n=5 for DB mouse types and n=3 for OB mouse types. DB/DB mice had significantly elevated plasma glucose, which was reduced while on COOH treatment. Error bars represent SEM. *P<0.0001

Figure 2A Phenylephrine induced contraction of SMA from db and ob mice The PE induced contraction was normalized to 120 mM KCl. The data are expressed as mean ± SEM. There are no significant differences between treated and untreated animals observed.

Figure 2B Maximal acetylcholine induced relaxation in SMA precontracted with phenylephrine. The data are expressed as mean ± SEM. *P<0.005 db/db and db/db crossover compared to db/db treated.

Figure 2C Sodium nitroprusside-induced relaxation following contraction with phenylephrine The data are expressed as mean ± SEM. No significant differences between treated and untreated animals are observed.

Figure 3 Quantitative PCR for eNOS mRNA in aorta and mesentery isolated from animal groups as labeled. n=5 in DB mice and n=3 in ob mouse types. Error bars represent one standard deviation from the mean.

Figure 4 Western blot of eNOS protein in thoracic aorta. Aortic tissue homogenates were loaded for equivalent total protein. A single band is observed at 140 kD in all samples. Left hand lane shows positions of molecular weight standards. Second and third lanes show aortic tissue from db/+ animals untreated (c-) and treated (c+) with COOH. Third and fourth lanes show aortic tissue from db/db animals: untreated (d-) and treated (d+) with COOH.

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	Mouse Weight (g)	*sem*	*Total Cholesterol (mM)*	*sem*	*Triglycerides (mM)*	*sem*	*MDA/ Cholesterol*	*sem*	*# Animals*
DB/+	**30.0**	**1.4**	**1.81*** **	**0.06**	**1.18**	**0.11**	**4.94**	**0.58**	**5**
DB/+ treated	**32.0**	**1.3**	**1.35*** **	**0.08**	**0.94**	**0.08**	**5.20**	**0.58**	**5**
DB/DB	**51.5**	**1.6**	**2.77**	**0.11**	**2.11** **	**0.61**	**5.41**	**0.31**	**5**
DB/DB treated	**54.3**	**1.8**	**2.66**	**0.15**	**1.04** **	**0.20**	**4.59**	**0.46**	**5**
OB/+	**30.2**	**0.6**	**2.06**	**0.12**	**1.53***	**0.07**	**4.28**	**0.70**	**3**
OB/+ treated	**31.7**	**0.4**	**1.73**	**0.14**	**0.90***	**0.01**	**3.51**	**0.62**	**3**
OB/OB	**60.7**	**2.6**	**4.30**	**0.12**	**1.60##**	**0.21**	**5.30**	**1.10**	**3**
OB/OB treated	**65.4**	**2.8**	**3.90**	**0.22**	**0.77##**	**0.05**	**3.96**	**0.63**	**3**

Table 1. Characteristics of experimental groups: body weights and plasma lipid profiles of mice prior to sacrifice. Treatment with COOH decreased total cholesterol in the DB/+ (***; P<0.001) and triglycerides in all other animal groups (**; P< 0.05 ; *, P<0.01, ##; P<0.05). Malondialdehyde levels (MDA; a marker of lipid peroxidation) did not show statistically significant differences between groups when normalized to total cholesterol

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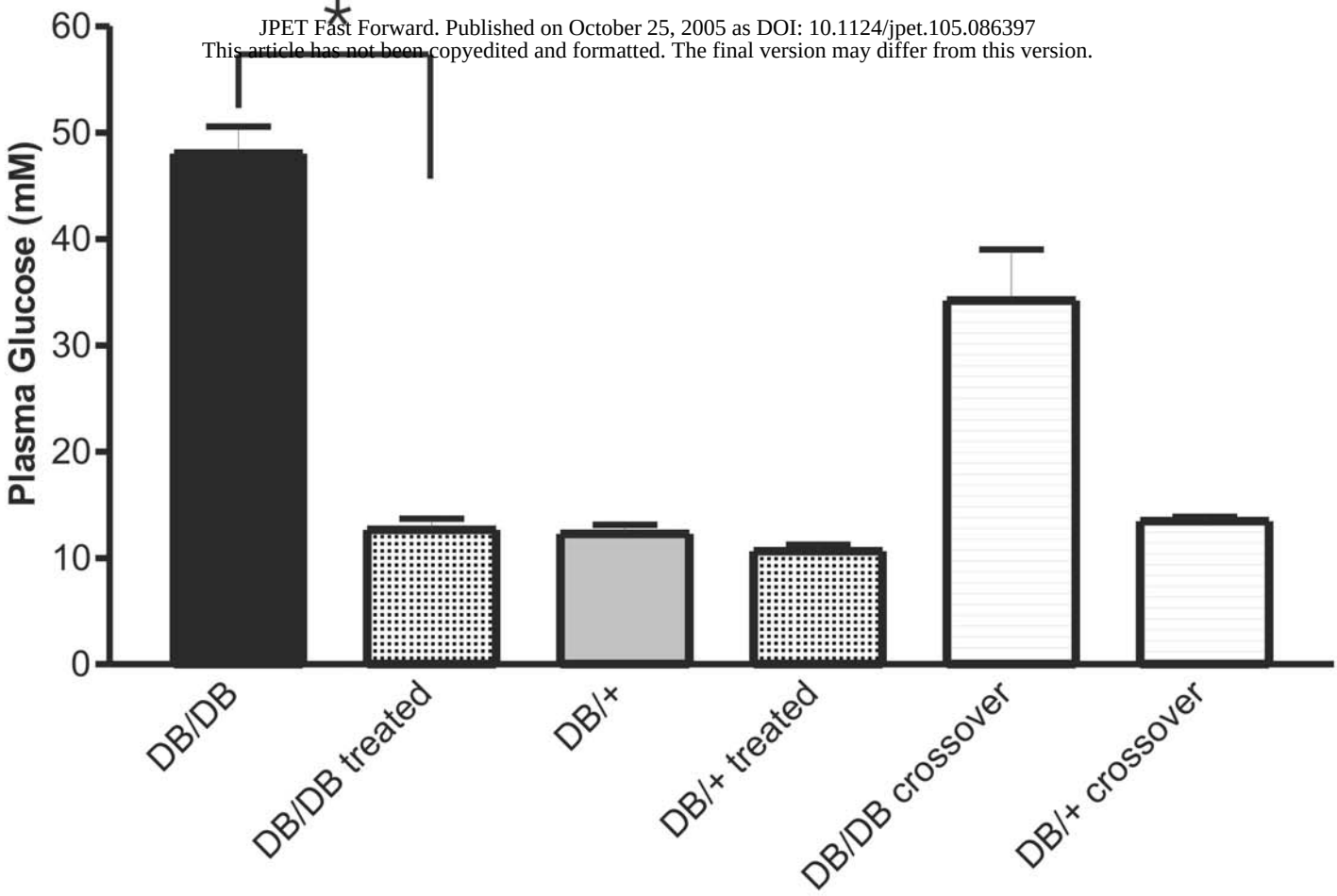

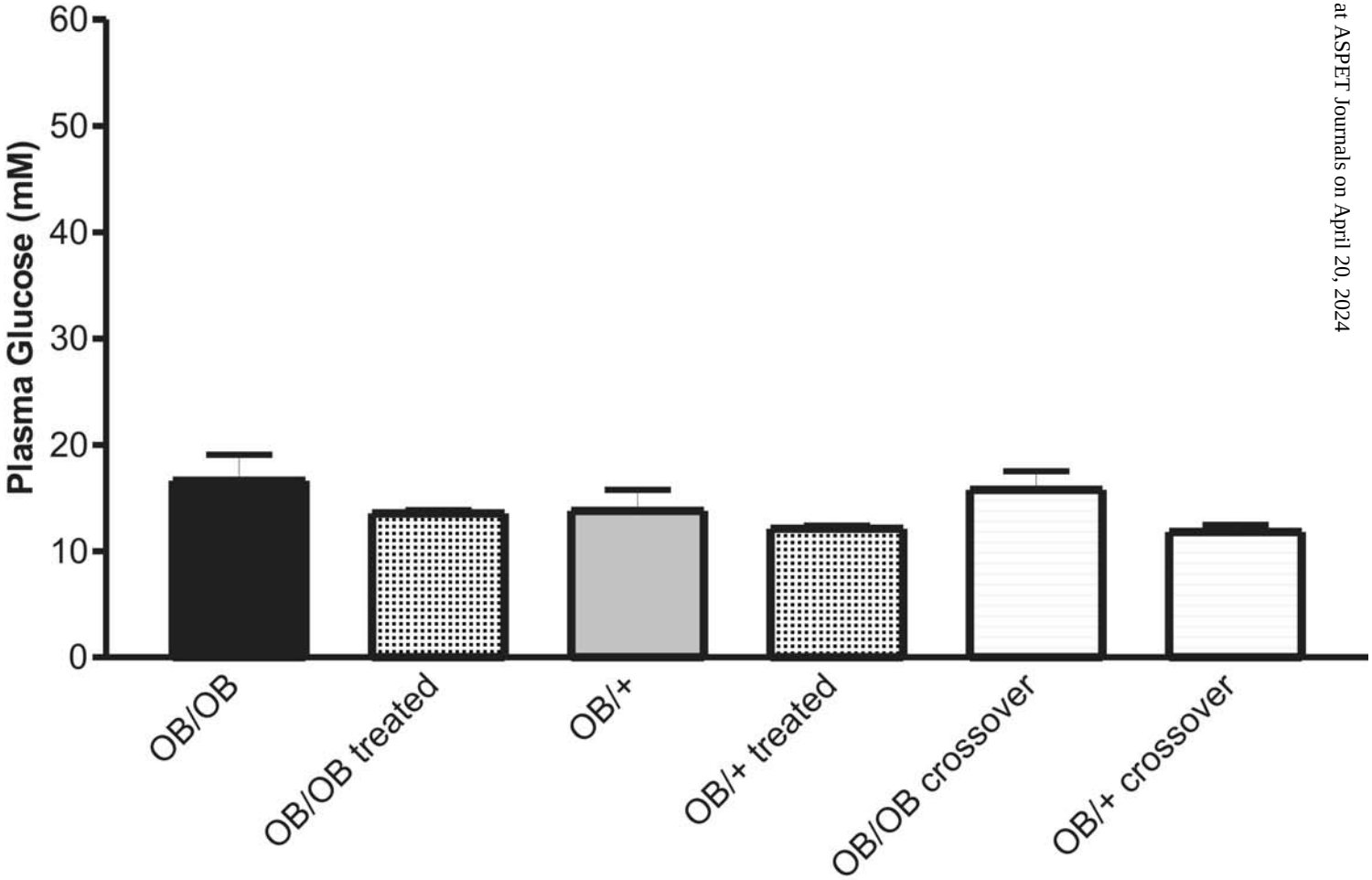

Figure 1

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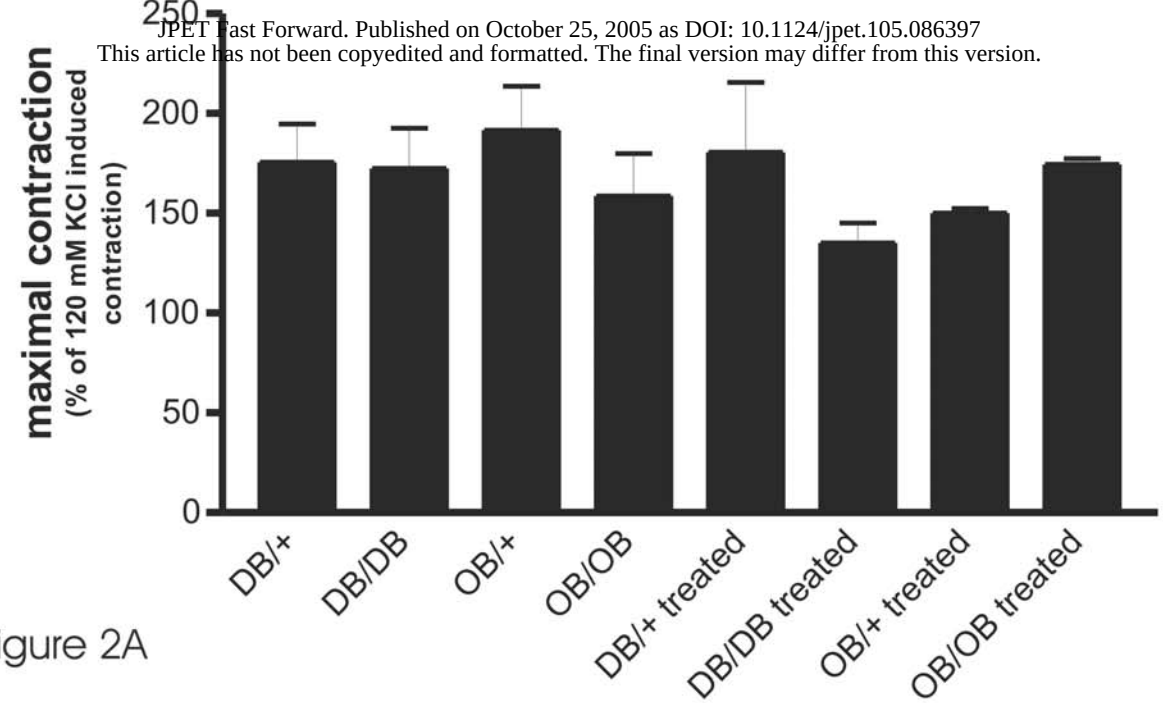

Figure 2A

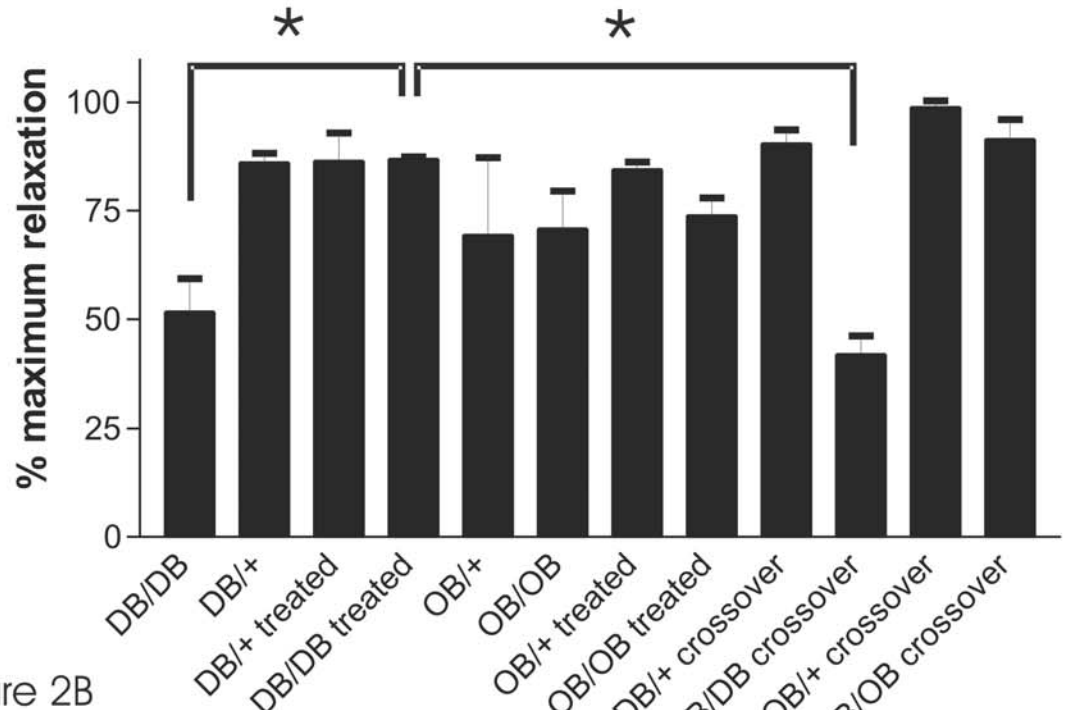

Figure 2B

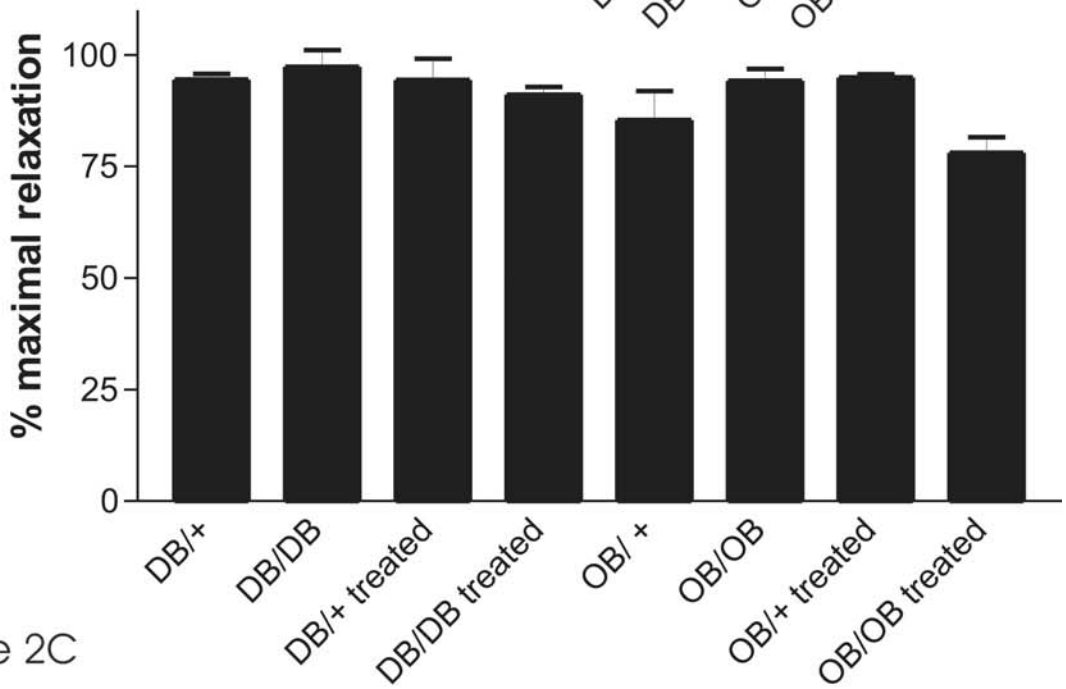

Figure 2C

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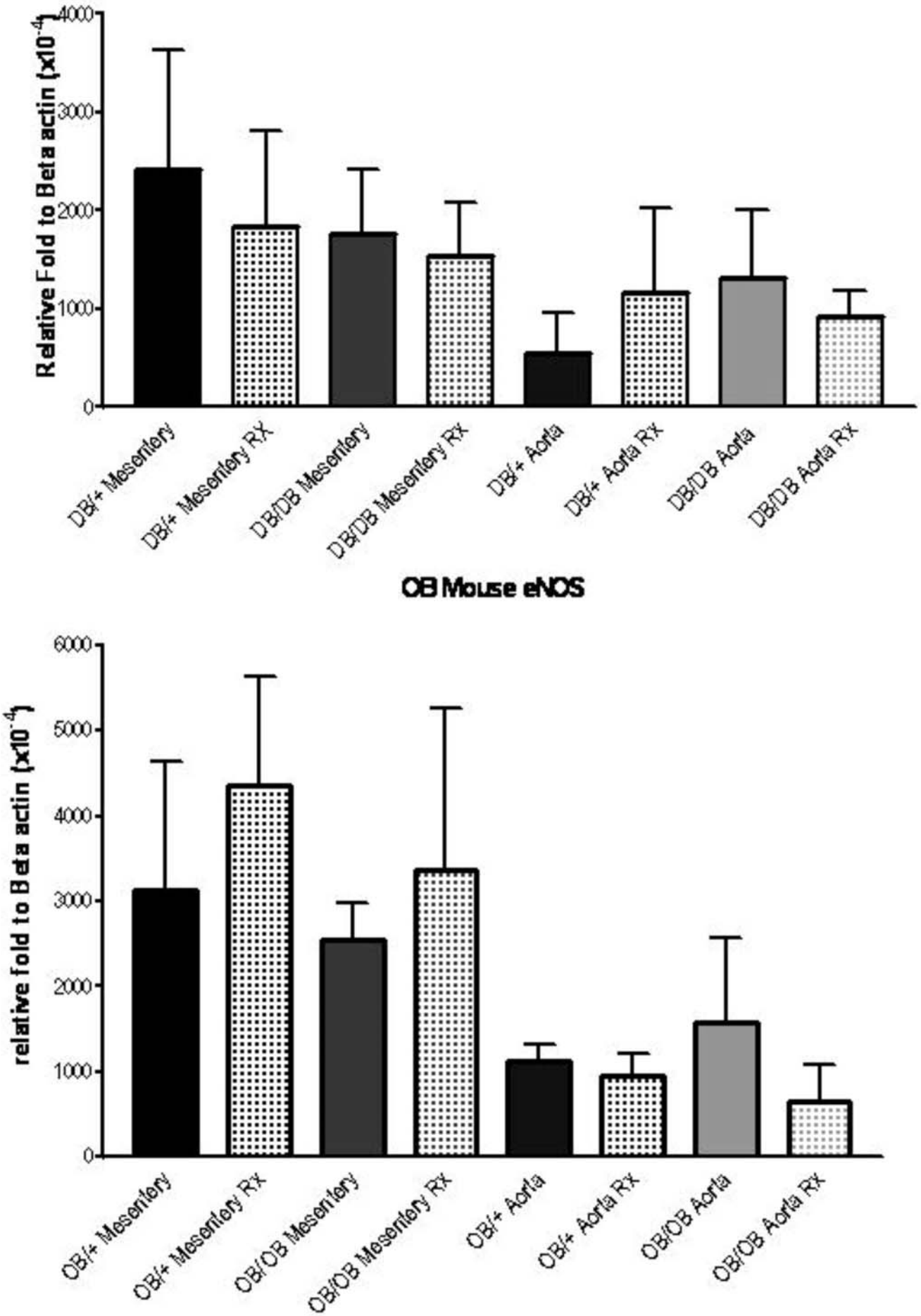

Figure 3

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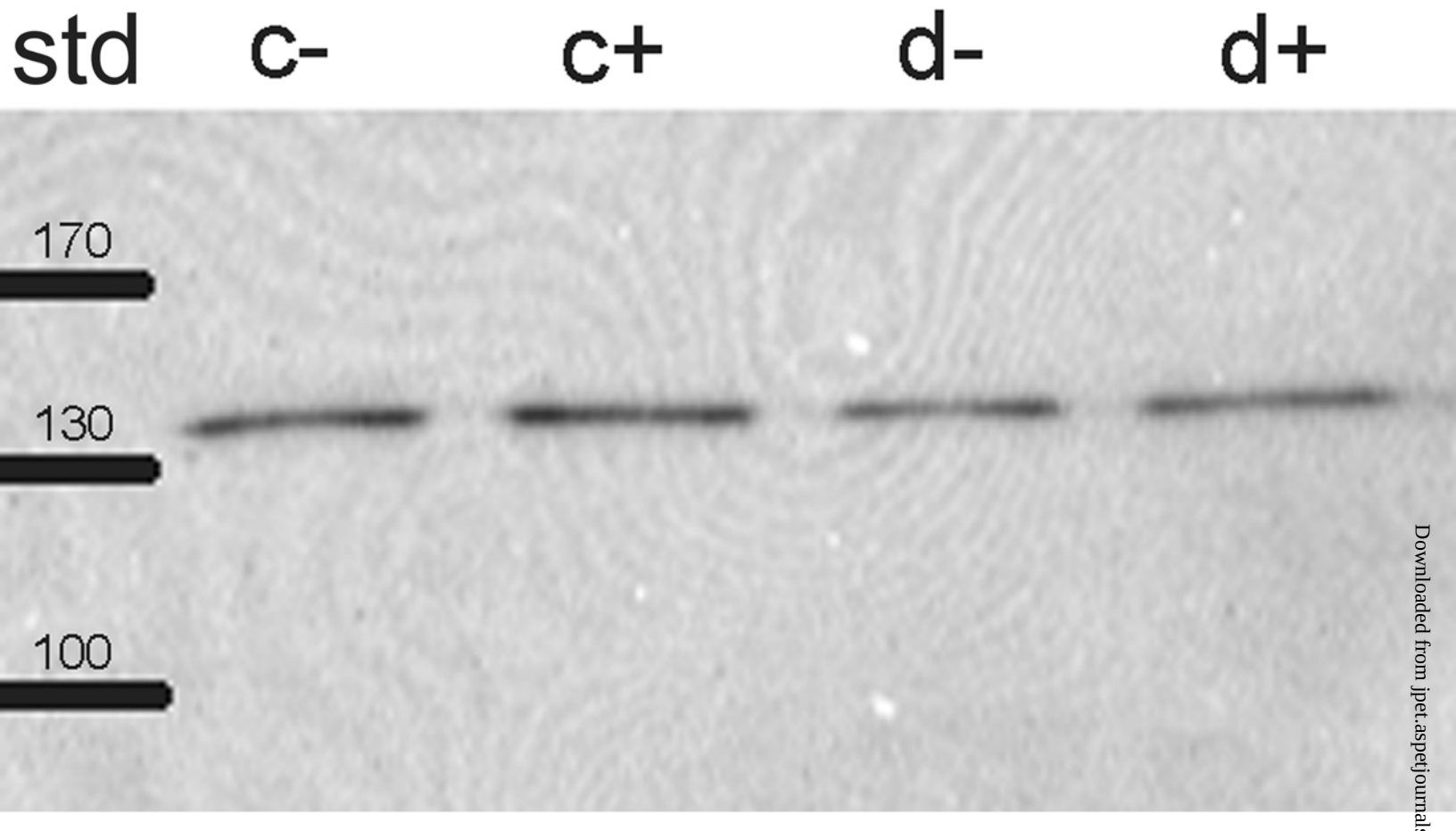

Figure 4